

Enhanced levels of Pis1p (phosphatidylinositol synthase) improve the growth of *Saccharomyces cerevisiae* cells deficient in Rsp5 ubiquitin ligase

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The Rsp5 ubiquitin ligase plays a role in many cellular processes including the biosynthesis of unsaturated fatty acids. The *PIS1* (phosphatidylinositol synthase gene) encoding the enzyme Pis1p which catalyses the synthesis of phosphatidylinositol from CDP-diacylglycerol and inositol, was isolated in a screen for multicopy suppressors of the *rsp5* temperature sensitivity phenotype. Suppression was allele non-specific. Interestingly, expression of *PIS1* was 2-fold higher in the *rsp5* mutant than in wild-type yeast, whereas the introduction of *PIS1* in a multicopy plasmid increased the level of Pis1p 6-fold in both backgrounds. We demonstrate concomitantly that the expression of *INO1* (inositol phosphate synthase gene) was also elevated approx. 2-fold in the *rsp5* mutant as compared with the wild-type, and that inositol added to the medium improved growth of *rsp5* mutants at a restrictive tem-

perature. These results suggest that enhanced phosphatidylinositol synthesis may account for *PIS1* suppression of *rsp5* defects. Analysis of lipid extracts revealed the accumulation of saturated fatty acids in the *rsp5* mutant, as a consequence of the prevention of unsaturated fatty acid synthesis. Overexpression of *PIS1* did not correct the cellular fatty acid content; however, saturated fatty acids (C_{16:0}) accumulated preferentially in phosphatidylinositol, and (wild-type)-like fatty acid composition in phosphatidylethanolamine was restored.

Key words: fatty acid, phosphatidylinositol synthase (Pis1p), phospholipid, Rsp5 ubiquitin ligase, *Saccharomyces cerevisiae*, multicopy suppressor.

INTRODUCTION

The regulation of metabolic lipid enzymes is an essential process that affects membrane lipid composition and has an impact upon many cell processes, such as cell growth, organelle function and response to stress etc. [1,2]. Therefore eukaryotes have developed complex mechanisms to regulate lipid biosynthetic pathways. Derregulation of lipid metabolism has been reported in many human diseases, including obesity and atherosclerosis, one of the diseases with the highest morbidity in developed countries [3].

The ratio of saturated to monounsaturated fatty acids that are incorporated into cell membranes contributes to fluidity of the membrane. In the yeast *Saccharomyces cerevisiae*, this ratio also affects mitochondrial inheritance [4] and stress responses [5]. The enzyme involved in fatty acid desaturation is the Δ -9 fatty acid desaturase, encoded by the essential *OLE1* gene, that converts saturated fatty acyl-CoA (palmitoyl- and stearyl-) to monounsaturated fatty acid species (palmitoleoyl- and oleoyl-) in an oxygen-dependent manner [6]. The regulation of *OLE1* expression is physiologically very important since unsaturated fatty acids contribute to 70–80% of the fatty acyl groups in membrane lipids. The expression of *OLE1* is regulated by nutrient fatty acids and molecular oxygen [7], and other physiological conditions, both at the transcriptional and mRNA stability levels [8,9]. Unsaturated fatty acid-dependent repression is mediated by FAR (fatty acid-regulated) elements [9] and hypoxic activation is mediated by LORE (low-oxygen response elements) [7].

Two highly similar and functionally related genes, *SPT23* (multicopy suppressor of tyrosine-induced mutations) and *MGA2*

[multicopy suppressor of *gam1* (*snf2*)], are required for the activation of *OLE1* transcription [10]. They are both functionally redundant since neither of the two genes is essential for viability, whereas the double *spt23Δmga2Δ* mutant is lethal [10]. This lethality is suppressed by the presence of oleic acid in the growth medium. Spt23p and Mga2p are produced as p120 precursors which are anchored as homodimers in the membrane of the endoplasmic reticulum via their C-terminal transmembrane domain [11,12]. When unsaturated fatty acids become limiting, the Spt23p and Mga2p precursors are ubiquitinated, and one of the dimer subunits is processed into a mature p90 form. Subsequently, p90 with the assistance of the chaperone complex Cdc49p/Ufd1p/Npl4p and the ubiquitin-proteasome pathway, is released from the p120 membrane-bound subunit and transported into the nucleus where it functions as a transcriptional activator of *OLE1* [12]. Mga2p is also essential for the hypoxic induction of *OLE1* expression and is a component of the LORE-bound complex [13]. Moreover, it regulates the half-life of the *OLE1* transcript by activating the exosomal 3'-5' exonuclease [14].

Ubiquitination of Spt23p and Mga2p is performed by the essential Rsp5 ubiquitin ligase [11], an E3 (ubiquitin ligase) enzyme of the ubiquitination pathway which transfers ubiquitin to lysine residues in protein substrates [15]. Rsp5p contains the C2, WW and catalytic Hect (homologous to E6-accessory protein C-terminus) domains. The C2 domain interacts with phospholipids [16], and three WW domains are involved in protein–protein interactions [15]. The WW3 domain is required for Rsp5p-induced processing of Spt23p [11]. Binding of the Rsp5p WW domains to Spt23p is directed via an LPKY motif, located near the C-terminal

Abbreviations used: CAT, chloramphenicol acetyltransferase; CDP-DAG, CDP-diacylglycerol; FAR, fatty acid-regulated elements; Hect, homologous to E6-accessory protein C-terminus; LORE, low-oxygen response element; *MGA2*, multicopy suppressor of *gam1* (*snf2*); PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; *PIS1*, PI synthase gene; PS, phosphatidylserine; SC, synthetic complete; SD, synthetic defined; *SPT23*, multicopy suppressor of tyrosine-induced mutations; ts, temperature sensitive; YEPD, yeast extract peptone dextrose.

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Table 1 Yeast strains used in the present study

Strain	Genotype	Source
T8-1D	<i>MATα SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3,112 his4-519</i>	[36]
TZ11	<i>MATα rsp5-17 ura3-1 SUP11 ade2-1 mod5-1 lys2-1 leu1 trp5 met4 rsp5-17</i>	[36]
TZ19	<i>MATα rsp5-9 ura3-1 SUP11 ade2-1 mod5-1 lys2-1 leu1 trp5 met4</i>	[31]
TZ29	<i>MATα rsp5-19 ura3-1 SUP11 ade2-1 mod5-1 lys2-1 leu2-3,112 his4-519</i>	Laboratory collection
MK1	<i>MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 <i>rsp5::HA-RSP5</i></i>	[37]
MK3	<i>MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 <i>rsp5::HA-rsp5-w2</i></i>	[37]
MK4	<i>MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 <i>rsp5::HA-rsp5-w3</i></i>	[37]
MK15	<i>MATα his3-Δ200 leu2-3, 112 ura3-5 lys2-801 trp1-1 <i>rsp5::HA-rsp5-19</i></i>	M. Kwapisz
Y25529	<i>Mat a/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0 <i>PIS1/pis1::kanMX4</i></i>	Euroscarf
PK10	<i>MATα SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3,112 his4-519 gal4::PIS1-CAT</i>	Present study
PK11	<i>MATα rsp5-19 ura3-1 SUP11 ade2-1 mod5-1 lys2-1 leu2-3,112 his4-519 gal4::PIS1-CAT</i>	Present study

transmembrane domain [17]. This interaction is necessary for Spt23p ubiquitination, processing, and *OLE1* activation [17]. A similar motif, found in Mga2p, is required for Mga2p binding and ubiquitination by Rsp5p; however, it is not required for Mga2p proteasome-dependent processing, leaving the function of this ubiquitination unknown [17]. Therefore by ubiquitinating Spt23p and Mga2p, Rsp5p is indirectly involved in the regulation of unsaturated fatty acid synthesis and, as a consequence, the *rsp5 Δ* mutant is lethal unless the growth medium is supplemented with oleic acid [11]. Rsp5p also affects many other cellular processes. The best known is the involvement of Rsp5p in endocytosis of plasma membrane transporters and pheromone receptors [18], and in other steps of vesicular trafficking [19]. It also affects transcription and nuclear transport of RNAs [20–23].

In the present study, we report the isolation of the *PIS1* gene [PI (phosphatidylinositol) synthase], encoding protein (EC 2.7.8.11) [24], as a multicopy suppressor of *rsp5* mutants. In this background, saturated fatty acids resulting from unsaturation preclusion tended to accumulate preferentially within the PI as a consequence of Pis1p overproduction.

EXPERIMENTAL PROCEDURES

Strains, media, growth and transformation conditions

Escherichia coli strain DH5 α *F'* *supE44 Δ lacU169* (Φ 80 *lacZ Δ M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* was used for cloning and plasmid propagation. The *S. cerevisiae* strains used are described in Table 1. Strain MK15 (from M. Kwapisz, Department of Genetics, IBB PAS, Warsaw) was constructed by the integration of YIpHA-*rsp5-19* into the MHY501 strain. Yeast strains containing the *PIS1-CAT* (chloramphenicol acetyltransferase) fusion gene integrated at the *GAL4* locus were created by transformation of T8-1D and TZ29 strains using a p911 plasmid digested by SacII and PvuI.

YEPD (yeast extract peptone dextrose), YEPG (yeast extract peptone galactose), SD (synthetic defined), SC (synthetic complete) and sporulation media were prepared and genetic manipulations were performed as described by Sherman [25]. YEPD medium was supplemented with 20 mg/l inositol or 1 % Tween-80 as indicated. SC-ura-inositol contained the same ingredients as SC media except for the presence of uracil and inositol. SD + cas medium contained the same ingredients as SD plus 1 % casamino acids, 20 mg/l tryptophan and 20 mg/l adenine.

Suppression of the ts (temperature-sensitive) phenotype of various mutants by the YEpHA-PIS1 plasmid was monitored by a drop test. Appropriate transformants were suspended in water, and serial 10-fold dilutions were plated on to YEPD. Growth was observed after a 2-day incubation at 34 or 37 °C as indicated.

Table 2 Primers used in the present study

Primer	Sequence
PIS1Not1	5'-ATTGCGGCCGCGAGTTCAATTCACTGGC CGTCGTT-3'
PIS1Not2	5'-ATTGCGGCCGCGCATCTTGACTATCACAC CTTTCCC-3'
PIS1M1	5'-GGGCTTGGATATTACTAGTTACTACATGC-3'
PIS1M2	5'-TACCGCTGGCTTGATGTG-3'

Plasmid constructions

The pMA109 multicopy plasmid expressing the *PIS1* promoter (*PIS1-lacZ*) [26], the integrative plasmid p911 expressing the *PIS1* promoter (*PIS1-CAT*) [27], and the multicopy plasmid pJH359 expressing the *INO1* (inositol phosphate synthase) promoter (*INO1-lacZ*) [28] were used.

Plasmid AJ2 bearing a fragment of the *DBF2* gene and genes *MRD1* and *PIS1* was isolated from the pFL44L [29] based (*URA3* marker) multicopy library, as complementing the growth defect of the TZ19 *rsp5-9* strain on YEPD at 37 °C [30]. AJ2 was digested with SacI and religated to obtain AJ2 Δ Sac bearing only the *PIS1* gene. To obtain YCpHA-PIS1, the EcoRI–EcoRI fragment, containing the *PIS1* promoter and an ATG codon, was transferred from the AJ2 plasmid and cloned into pUC19. A NotI site was introduced after the ATG codon by reverse PCR using specific primers (Table 2) for site-directed mutagenesis. The sequence of the plasmid after mutagenesis was confirmed. The NotI–NotI fragment encoding a triple HA tag from pBF30 [31], was cloned into the NotI site to construct the pPK1 plasmid. The EcoRI–SphI fragment of AJ2, containing a fragment of the *PIS1* gene was cloned into YCp33 [32]. The EcoRI–EcoRI fragment from pPK1 was then cloned into the EcoRI site. To obtain YEpHA-PIS1, the PvuII–SphI fragment of YCpHA-PIS1 containing the *HA-PIS1* gene was cloned into plasmid pFL44L. YEpHA-pis1(H114Y) was constructed by PCR mutagenesis. To this end the 750 bp fragment of *PIS1* was amplified using specific primers (Table 2), one containing a C \rightarrow T mutation yielding CAC(114)TAC. The PCR product was cloned into pGEM-T (Promega) to obtain pGEM-pis1 and its sequence was confirmed. The SpeI–SphI fragment of YEpHA-PIS1 was replaced by the SpeI–SphI fragment transferred from pGEM-pis1.

Cell extracts and immunoblot analyses

To study the level of Pis1p, cell extracts were prepared by alkaline lysis [33]. Proteins were resolved by SDS/PAGE and the HA–Pis1 and HA–Rsp5 tagged proteins were revealed using the monoclonal mouse anti-HA antibody 16B12 (BabCO). Rabbit anti-Hts1p antibody (a gift from T. Mason, Department of Biochemistry and Molecular Biology, University of Massachusetts, MA,

U.S.A.) was used as a control. CAT was visualized using a rabbit monoclonal anti-CAT antibody (Promega). Primary antibodies were detected with goat horseradish peroxidase-conjugated secondary antibodies (Dako) followed by enhanced chemiluminescence (Amersham Biosciences).

Lipid analyses

Lipid extracts of MK1 and MK15 strains bearing an empty plasmid or plasmid YEpHA-PIS1 were prepared from approx. 2×10^9 (phospholipids) or 2×10^8 (total fatty acids) yeast cells, grown as described. Cells were harvested, washed with distilled water, suspended in 1 ml of cold water and shock-frozen. They were lysed by vigorous shaking with 500 μ l of glass beads (diameter 0.3–0.4 mm; Sigma) using Mini-BeadBeaterTM (Biospec Products) for 1 min at 5000 rev./min. Cell lipids were extracted with chloroform/methanol (2:1, v/v). The organic phase was evaporated and the lipids were dissolved in 100 μ l of chloroform/methanol (1:1). Phospholipids were resolved on precoated LK5 silica gel plates (Whatmann) using chloroform/ethanol/water/triethylamine (30:35:7:35, by vol.) as the mobile phase. They were visualized under UV after vaporization with a primuline solution [0.05 mg/ml in acetone/water (80:20, v/v)] and identified using appropriate standards. Phospholipids were scraped from the plate. Fatty acid methyl esters either of total fatty acids or of phospholipids were obtained as described by Ferreira et al. [34]. Briefly, cell lipid extracts or the various phospholipids were eluted from the silica with chloroform/methanol/water (5:5:1, by vol.) and transesterification was carried out by heating the eluate at 50°C for 16 h in 2% (v/v) H₂SO₄ in dry methanol. The resulting fatty acid methyl esters were extracted with hexane and analysed by gas chromatography using a 25 mm \times 0.32 mm AT-1 capillary column (Alltech), with heptadecanoicmethyl ester as the standard. Fatty acids were quantified and used to calculate the amount of lipid in each class. The specific lipid content was finally expressed as a percentage of total lipids. For the data presented in Figures 7 and 8 the significance of differences between the lipid species was assessed by the ANOVA test, using the GraphPad Prism software ($P < 0.05$). This test is appropriate for comparing means for three or more variables.

β -Galactosidase assays

β -Galactosidase assays were performed as described previously [35]. β -Galactosidase activity A (given as *O*-naphthol nmol/min per mg of protein, measured at 420 nm) was calculated as follows: $A = (A_{420} \times 100 \times 1700) / (4.5 \times V \times c \times t)$, where *V* is the volume of the protein extract (ml), *c* is the protein concentration (mg/ml) and *t* is the incubation time (min) at 30°C.

RESULTS

PIS1 is a multicopy suppressor of *rsp5* mutations

Rsp5 ubiquitin ligase takes part in many cellular processes. To better understand the role of Rsp5p and its influence on various biosynthetic and regulatory pathways, a screen of multicopy *rsp5-9* suppressors was performed [30]. Among the many suppressor genes encoding ubiquitin, the *CCT6* gene encoding one of the subunits of the Cct chaperonin was identified [30]. Here we analysed a plasmid isolated in the same screen, bearing a fragment of *DBF2* along with the *MRD1* and *PIS1* genes. Deletion analyses revealed that the *PIS1* gene encoding Pis1p was responsible for the suppression of the *rsp5-9* ts growth defect. This suppression was allele-non specific since the overexpressed *PIS1* gene suppressed all of the alleles tested: *rsp5-w2*, *rsp5-9*, *rsp5-19*, *rsp5-w3* and

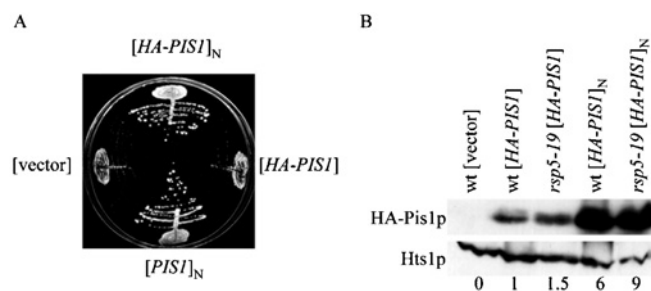


Figure 1 Overexpression of *PIS1* from a multicopy plasmid improves the growth of the *rsp5-19* mutant at an elevated temperature

(A) Growth of the *rsp5-19* expressing vector, [vector]; HA-PIS1 from the single copy plasmid, [HA-PIS1]; *PIS1* from the multicopy plasmid, [PIS1]_N, and HA-PIS1 from the multicopy plasmid, [HA-PIS1]_N, on YEPD after 2 days incubation at 34°C. (B) Western blot analysis of cellular extracts of the wild-type (wt) and *rsp5-19* strains bearing the vector, the single copy plasmid YCpHA-PIS1 or the multicopy plasmid YEpHA-PIS1 was performed. Anti-HA antibody and anti-Hts1p antibody (protein loading control) was used. The numbers below the blot represent the ratio of the HA-Pis1p level in each strain compared with the level of HA-Pis1p in the wild-type strain expressing the YCpHA-PIS1 plasmid.

rsp5-17 (expressed on two different genetic backgrounds), which contained point mutations causing amino acid substitutions in various WW domains (*rsp5-w2*, W359F, P362A in WW2; *rsp5-9*, P418S; *rsp5-19*, P418L; *rsp5-w3*, W415F and P418A in WW3) or the Hect domain (*rsp5-17/mdp1-1*; P784T) and conferred the ts growth defect [31,36,37] (Figure 1A, and results not shown). Overproduction of *PIS1* also allowed germination and several divisions of *rsp5Δ* spores at 23°C on complete medium without oleic acid addition (results not shown). Furthermore, the HA-PIS1 gene on the multicopy plasmid that expressed N-terminally tagged Pis1p was also active as a suppressor of *rsp5-19* (Figure 1A). This YEpHA-PIS1 plasmid fully complemented the normally lethal *pis1Δ* allele [24]. Complementation was tested by transformation of heterozygous *PIS1/pis1Δ* diploid, sporulation and tetrad analysis (results not shown). However, the HA-PIS1 tagged version expressed from a single copy plasmid did not suppress the *rsp5-19* mutation (Figure 1A), indicating that the copy number of the plasmid, and therefore high cellular levels of Pis1p are probably important for suppression.

PIS1 suppression of *rsp5* is related to cellular level of Pis1p

Cellular levels of Pis1p were analysed by Western blotting in the wild-type and *rsp5-19* backgrounds using HA-PIS1 expressed from either single or multicopy plasmids. The respective transformants were grown at 30°C and protein extracts were prepared. There was a 6-fold higher level of Pis1p in strains bearing the multicopy plasmid YEpHA-PIS1 as compared with strains bearing the single copy plasmid YCpHA-PIS1 (Figure 1B). In addition, the level of Pis1p was about 1.5-fold higher in the *rsp5-19* mutant strain than in the wild-type strain. These results indicate that a very high level of Pis1p is important for suppression and that *PIS1* expression is elevated in the *rsp5-19* background.

To verify whether *PIS1* expression is regulated at the translational or transcriptional level in the *rsp5* strain, transcriptional reporter fusions were introduced into wild-type and *rsp5-19* backgrounds using a multicopy plasmid bearing a *PIS1-lacZ* fusion gene (pMA109) or an integrative plasmid bearing a *PIS1-CAT* fusion reporter gene (p911). β -galactosidase activity was measured and the level of CAT was determined by Western blotting (Figure 2, A and B). At the transcriptional level *PIS1* was increased approx. 2-fold in *rsp5-19* mutant strains as compared with the wild-type strain, whatever the reporter system used, when cells

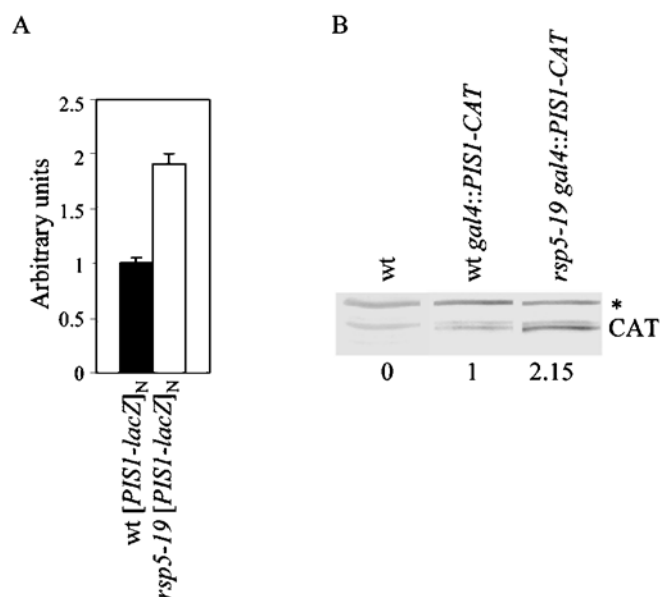


Figure 2 Expression of the *PIS1* gene is increased in the *rsp5-19* mutant strain

(A) Wild-type (wt) and *rsp5-19* strains expressing [*PIS1-lacZ*]_N from the multicopy plasmid were grown on SD + cas medium, extracts were prepared and the activity of β-galactosidase was compared. Values are means ± S.D. of at least 3 independent experiments. (B) Western blot analysis of wild-type (wt) and *rsp5-19* strains expressing the *PIS1-CAT* cassette integrated into the *gal4* locus. A specific anti-CAT antibody was used. The nonspecific protein (*) which was also recognized by the anti-CAT antibody was used as a loading control. Quantification of CAT is shown below the blot and represents the ratio of CAT level in *rsp5-19* over the level of CAT in the wild-type strain expressing *PIS1-CAT*.

were grown on glucose medium (Figure 2, A and B and results not shown). It was also higher in *rsp5* cells than in wild-type cells grown in a galactose-containing medium (results not shown), a carbon source known to induce *PIS1* transcription [26]. From these results, we conclude that *rsp5-19* affects the regulation of *PIS1* expression at the transcription level.

Pis1p catalytic activity is essential for suppression

To investigate if the catalytic activity of Pis1p is essential for suppression, a multicopy plasmid containing the *HA-pis1(H114Y)* mutant allele was constructed by site-directed mutagenesis. This allele causes an amino acid substitution in Pis1p previously shown to result in almost complete (0.2% of wild-type levels) loss of catalytic activity [38]. Interestingly, this plasmid did not suppress the growth defects of the *rsp5-19* strain (MK15) even though the Pis1p(H114Y) was synthesized at similar levels as Pis1p (Figure 3, A and B). Therefore a high level of active Pis1p overexpression is necessary for suppression of *rsp5-19* and it can be proposed that overproduction of PI, the product of Pis1p, may help *rsp5* cells to grow at elevated temperatures.

Addition of inositol to the medium improves growth of the *rsp5-19* strain, and expression of the *INO1* gene is upregulated in the *rsp5-19* strain

In PI synthesis Pis1p utilizes CDP-DAG (CDP-diacylglycerol) and inositol as substrates [2]. Supplementation of yeast cells with inositol increases the cellular level of PI because the *K_m* of Pis1p for this substrate is much higher than the intracellular inositol concentration resulting from endogenous synthesis [39]. Therefore we tested the growth of the *rsp5-19* strain at 34 °C on a

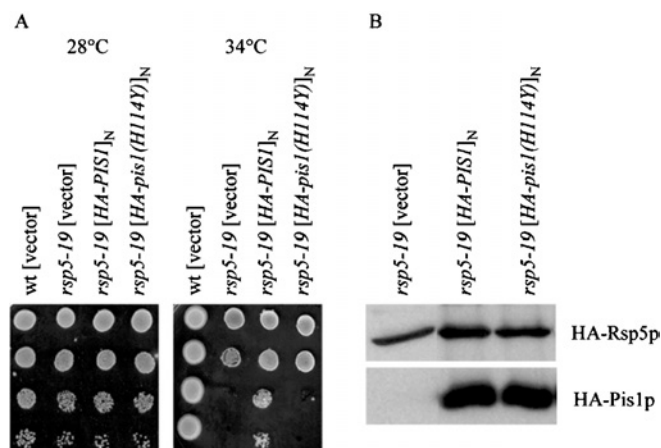


Figure 3 YEpHA-*pis1(H114Y)* does not suppress the *rsp5-19* ts phenotype

(A) Wild-type (wt) and *rsp5-19* strains were transformed with vector or multicopy plasmids carrying HA-*PIS1* or HA-*pis1(H114Y)*. Serial dilutions (1:10-fold) of transformants were spotted on to YEPD plates and grown at the indicated temperatures for 2 days. (B) Western blot analysis of cell extracts of the *rsp5-19* strain bearing the vector or the multicopy plasmids, YEpHA-*PIS1* or YEpHA-*pis1(H114Y)*. Anti-HA antibody recognizing HA-Pis1p and HA-Rsp5p (loading control) was used.

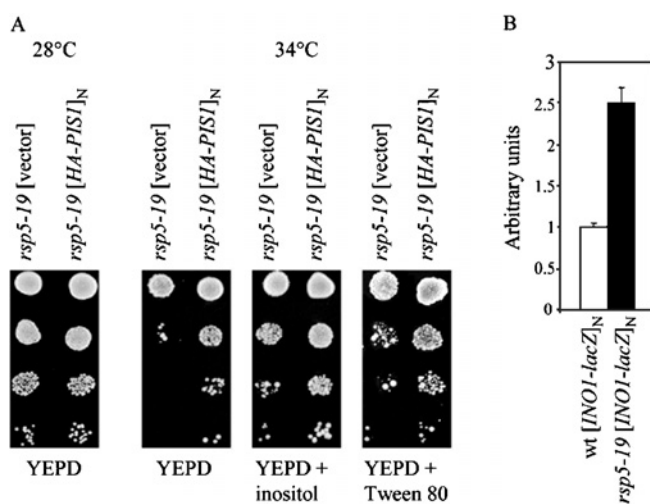


Figure 4 Addition of inositol to the medium improves growth of the *rsp5-19* strain and expression of *INO1* is increased in the *rsp5-19* strain

(A) The *rsp5-19* strain was transformed with the vector or the multicopy plasmid carrying HA-*PIS1*. Serial dilutions (1:10 steps) of transformants were spotted on to YEPD, YEPD + inositol (20 mg/l), YEPD + Tween-80 (1%) and allowed to grow at the indicated temperatures for 3 days. (B) Wild-type (wt) and *rsp5-19* strains expressing *INO1-lacZ* from the multicopy plasmid were grown on SC-ura-inositol medium, extracts were prepared and the activity of β-galactosidase was compared. Values are means ± S.D. for at least 3 independent experiments.

medium supplemented with 20 mg/l inositol. This temperature was chosen, since it is the optimal temperature for *rsp5-19* suppression by *PIS1* in the MK15 strain. As shown in Figure 4(A), inositol that was added to the medium improved the growth of the *rsp5-19* mutant at a restrictive temperature, to the same extent as overexpression of *PIS1*, and in a similar way as did medium supplemented with Tween-80 that was used as source of unsaturated fatty acid (namely oleic acid). Since a high inositol concentration in the medium results in increased PI synthesis [39], we postulate that the addition of inositol may restore the growth of *rsp5-19* in a similar manner as does overexpression of *PIS1*.

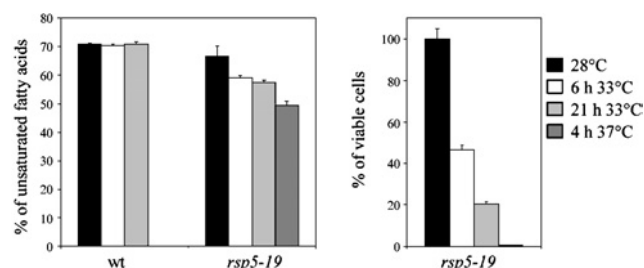


Figure 5 Accumulation of saturated fatty acids in *rsp5-19* correlates with cell viability at elevated temperatures

Wild-type (wt) and *rsp5-19* cells were grown to stationary phase at 28 °C, then diluted to an attenuance of approx. 0.5, incubated for 2 h at 28 °C, 6 h at 33 °C, 21 h at 33 °C or 4 h at 37 °C (*rsp5-19* only). The level of unsaturated fatty acids was measured. Values are means \pm S.D. for at least 3 independent experiments. Samples from cultures were plated on to YEPD medium, incubated for 2 days and the colonies counted.

In the cell, inositol phosphate is produced by inositol phosphate synthase encoded by *INO1* [2]. Since addition of inositol to the medium improved the growth of the *rsp5-19* strain, we postulated that the expression of *INO1* could be increased in the *rsp5-19* strain, as is the expression of *PIS1*. The level of *INO1* transcription was analysed using the multicopy plasmid pJH359 expressing a *INO1-lacZ* fusion reporter gene in cells grown in SC-uracil. The β -galactosidase activity was 2.5-fold higher in the *rsp5-19* strain compared with the wild-type strain (Figure 4B) indicating that *INO1* transcription was indeed increased in the *rsp5-19* background. Thus the expression of both *INO1* and *PIS1*, encoding two enzymes in the PI biosynthetic pathway, is enhanced in the *rsp5-19* strain.

Overexpression of *PIS1* does not restore wild-type fatty acid patterns in the *rsp5-19* strain

An essential function of Rsp5 ubiquitin ligase is to regulate the expression of *OLE1*, encoding fatty acid desaturase [11,12,17]. Thus one of the effects of the *rsp5-19* mutation might be to lower unsaturated fatty acid levels and consequently to induce accumulation of saturated fatty acids in the cell. We therefore studied whether accumulation of saturated fatty acids correlated with the survival of the *rsp5-19* strain at elevated temperatures. In *S. cerevisiae*, C_{16:1} (palmitoleic acid) and C_{18:1} (oleic acid) are the main unsaturated species, whereas C_{16:0} (palmitic acid) and C_{18:0} (stearic acid) are the most abundant saturated fatty acids [40]. Under normal aerobic conditions fatty acids are mainly unsaturated in yeast, since the percentage unsaturation ratio (i.e. the unsaturated forms as a percentage of total fatty acids) of

major complex lipids is about 70–80% [40]. In the experiment presented in Figure 5, *rsp5-19* cells were grown at the permissive temperature (28 °C) for 16 h and transferred to 33 °C for 6 or 21 h or for 4 h to the more restrictive temperature, 37 °C. Interestingly, the percentage of unsaturated fatty acids in the *rsp5-19* strain dropped from 65% at 28 °C to values below 60% when cells were incubated at 33 °C, and to 50% when cells were grown at 37 °C (Figure 5). Simultaneously, survival dropped to 45 or 20% when cells were incubated at 33 °C for 6 or 21 h respectively. No cells were viable after incubation for 4 h at 37 °C. A control experiment in which wild-type cells were grown at 28 °C and transferred to 33 °C for 6 or 21 h constantly, showed 70% unsaturated fatty acids. Cells require unsaturated fatty acids for survival [11] and saturated fatty acid accumulation is deleterious to yeast [4]. The results of our experiments suggest a correlation between the accumulation of saturated fatty acids and *rsp5-19* cell lethality at 33 °C. This is supported by the observation that cell growth can be restored at this temperature when Tween-80, used as source of unsaturated fatty acid (namely oleic acid), is added to the medium (Figure 4A); hence, this phenomenon, similar to the recently demonstrated effect of *rsp5* on nuclear transport of RNA [21], could provide at least a partial explanation for the lethality of *rsp5-19* cells at elevated temperatures.

A possible explanation for *rsp5-19* suppression by overexpression of *PIS1* was the restoration of a wild-type-like unsaturated to saturated fatty acid ratio. To test this possibility, the total fatty acid content was determined in the *rsp5-19* mutant strain, in the *rsp5-19* mutant overexpressing *PIS1* and in the congenic wild-type strain, used as a reference. Cells were grown at 28 °C and transferred to 33 °C for 21 h. These conditions were chosen after growth and viability tests establishing the optimum suppressive effect of *PIS1* overexpression on the *rsp5-19* mutant. Total unsaturated fatty acid levels and the amount of individual fatty acids in these three strains were determined. As shown in Figure 6, at a non-permissive temperature the *rsp5-19* mutation led to an increase in saturated fatty acids (C_{16:0} and C_{18:0}) at the expense of unsaturated forms, as compared with the wild-type strain. Interestingly, this fatty acid composition was observed in *rsp5-19* cells, irrespective of *PIS1* overexpression (Figure 6). From these results, we conclude that overexpression of *PIS1* does not restore the wild-type fatty acid composition in *rsp5-19*.

The *rsp5-19* mutation and *PIS1* overexpression in the wild-type strain affect the PI/PE (phosphatidylethanolamine) ratio

We expected overexpression of *PIS1* to result in PI accumulation in the cells. To test this, phospholipid analyses were performed in wild-type and *rsp5-19* cells, with or without *PIS1* overexpression.

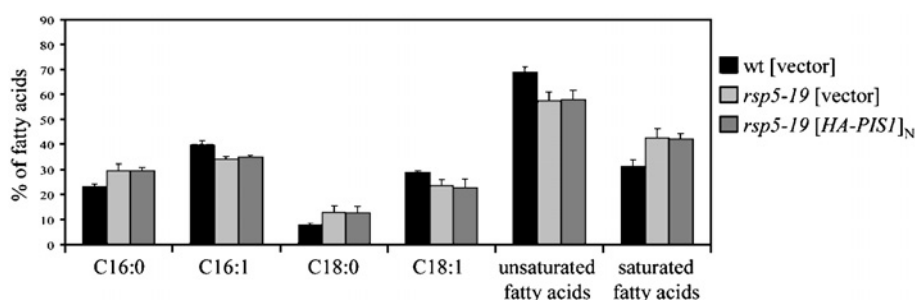


Figure 6 Overexpression of *PIS1* does not change the fatty acid distribution in *rsp5-19* cells

The *rsp5-19* strain transformed with the vector or the multicopy plasmid bearing *HA-PIS1*, and wild-type strain (wt) transformed with the vector were grown at 33 °C for 21 h. The levels of various fatty acids were determined and are presented as a percentage of total fatty acids. Values are means \pm S.D. for at least 3 independent experiments.

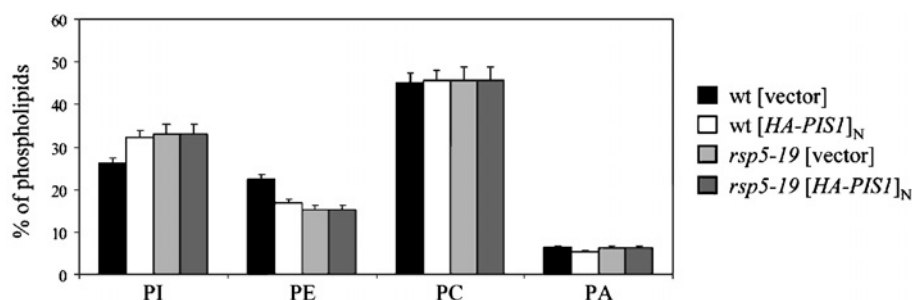


Figure 7 The *rsp5-19* mutation, and *PIS1* overexpression affect the PI/PE ratio

Wild-type (wt) and *rsp5-19* strains both transformed with vector or plasmid YEHA-*PIS1* were grown at 33°C for 21 h. The level of each phospholipid was determined and is presented as a percentage of total phospholipids. Values are means \pm S.D. for at least 3 independent experiments. Significance of differences was examined by ANOVA ($P < 0.05$, $n = 3$).

Indeed, the level of PI increased in the wild-type strain overexpressing *PIS1* (compare wild-type [vector] with wild-type expressing the multicopy plasmid [HA-*PIS1*]_N in Figure 7). Surprisingly, PI was increased to the same extent in the *rsp5-19* mutant (*rsp5-19* [vector], Figure 7). This was possibly due to the higher transcriptional activation of genomic *PIS1* observed in this background (see Figure 2). Furthermore, the overexpression of *PIS1* from a multicopy plasmid did not cause a further increase in PI levels in the *rsp5-19* strain (*rsp5-19* [HA-*PIS1*]_N, Figure 7). All strains with increased levels of PI showed decreased levels of PE. This observation is to be expected since *Pis1p* competes for the common substrate CDP-DAG with *PS* (phosphatidylserine) synthase (*Cho1p*), whose product, *PS*, is directly converted into *PE* by *PS* decarboxylase (*Psd1p* and *Psd2p*) [2]. The amounts of *PC* (phosphatidylcholine) and *PA* (phosphatidic acid) were similar in all strains analysed (Figure 7). It should be noted that since the amount of *PS* was very low under our experimental conditions, it could not be determined. These results indicate that overexpression of *PIS1* causes an increase in the amount of PI at the expense of *PE* in the wild-type strain and that the increased PI level in *rsp5-19* is possibly a regulatory adaptation of cells to physiological changes caused by this mutation. However, the presence of additional copies of the *PIS1* gene in *rsp5-19*, which improves the growth of this mutant at 33°C, does not result in a further increase in the amount of PI, implying that an increased PI level per se is not responsible for suppression.

Overexpression of *PIS1* in the *rsp5-19* mutant favours saturated fatty acid incorporation into PI and restores wild-type fatty acid composition in PE

The next step towards understanding the mechanism of *rsp5* suppression by *PIS1* was to investigate whether the *rsp5-19* mutation and/or overexpression of *PIS1* might affect saturated fatty acid distribution among the various phospholipids. To this end, the fatty acid composition of phospholipids was determined in the wild-type [vector], wild-type [HA-*PIS1*]_N, *rsp5-19* [vector] and *rsp5-19* [HA-*PIS1*]_N strains, grown for 21 h at 33°C. Overexpression of *PIS1* in wild-type strain essentially did not change the fatty acid composition of any of the phospholipids analysed: PI, PE, PC and PA (Figure 8). Thus whereas overexpression of *PIS1* in the wild-type background significantly affects the PI/PE ratio (see Figure 7), it does not influence the overall fatty acid content of these phospholipids. Strikingly, the *rsp5-19* mutation resulted in a specific, dramatic increase of saturated fatty acyl chains at the expense of unsaturated forms in *PE*, and to a lesser extent in *PC* (*rsp5-19* [vector], Figure 8). Overexpression of *PIS1* in this background (*rsp5-19* [HA-*PIS1*]_N, Figure 8), induced recovery

of the unsaturation ratio in *PE* to a level very similar to that in the wild-type strain. Importantly, the decrease in *C*_{16:0} content in *PE*, observed when *PIS1* is overexpressed, could be related to a low but significant increase in *C*_{16:0} content in *PI* at the expense mainly of the *C*_{16:1} species. Altogether, these results suggest that the *rsp5-19* mutation induces saturated fatty acid accumulation in *PE*, a phenomenon that can be fully suppressed by overexpression of *PIS1*, due to rerouting of these saturated forms towards *PI*.

DISCUSSION

The synthesis of fatty acids, phospholipids, dolichols and sterols in response to nutrient availability and cellular needs is highly regulated and is co-ordinated to build and maintain properly composed cellular membranes [2,41]. In particular, unsaturated fatty acids are crucial determinants of membrane fluidity since they regulate packing of phospholipids. Hence, the main enzyme involved in fatty acid desaturation in yeast, the Δ -9 fatty acid desaturase *Ole1p* is essential for growth, unless the cells are cultivated in the presence of an exogenous unsaturated fatty acid such as oleate. *Rsp5* ubiquitin ligase is involved in the regulation of *OLE1*. Consequently, *rsp5*Δ mutants display an auxotrophy for oleate [11]. This shows that when a decrease in unsaturated fatty acid synthesis is combined with other effects related to *rsp5* mutations it has a pernicious effect on growth.

In a search for multicopy suppressors of *rsp5* mutations, we isolated the *PIS1* gene, encoding *PI* synthase. *Pis1p* is essential in yeast since it is the only enzyme that catalyses the synthesis of *PI* from CDP-DAG and inositol. We demonstrated that the loss of viability of the *rsp5-19* mutant at increasing non-permissive temperatures correlates with the decrease in the unsaturation/saturation ratio of fatty acids i.e. accumulation of saturated forms at the expense of unsaturated species. However, overexpression of *PIS1* did not result in recovery of wild-type cellular fatty acid composition: in the presence of high levels of *Pis1p*, the *rsp5-19* mutation still caused a dramatic decrease in the level of unsaturated fatty acids. This result was somewhat expected if one considers the catalytic function of *Pis1p*, and suggests that the suppression of *rsp5* mutations by overexpression of *PIS1* is directly related to *PI* biosynthesis. Several observations are consistent with this assumption. First, we demonstrated that the catalytic activity of *Pis1p* is essential for suppression. Second, the *rsp5-19* mutation was suppressed by the addition of inositol into the medium; it had already been shown that cells grown in medium supplemented with inositol synthesize more *PI* [39]. Third, we show that *PIS1* is upregulated on the *rsp5-19* background (with a 2-fold increase in transcription and a 1.5-fold increase in protein-amount compared

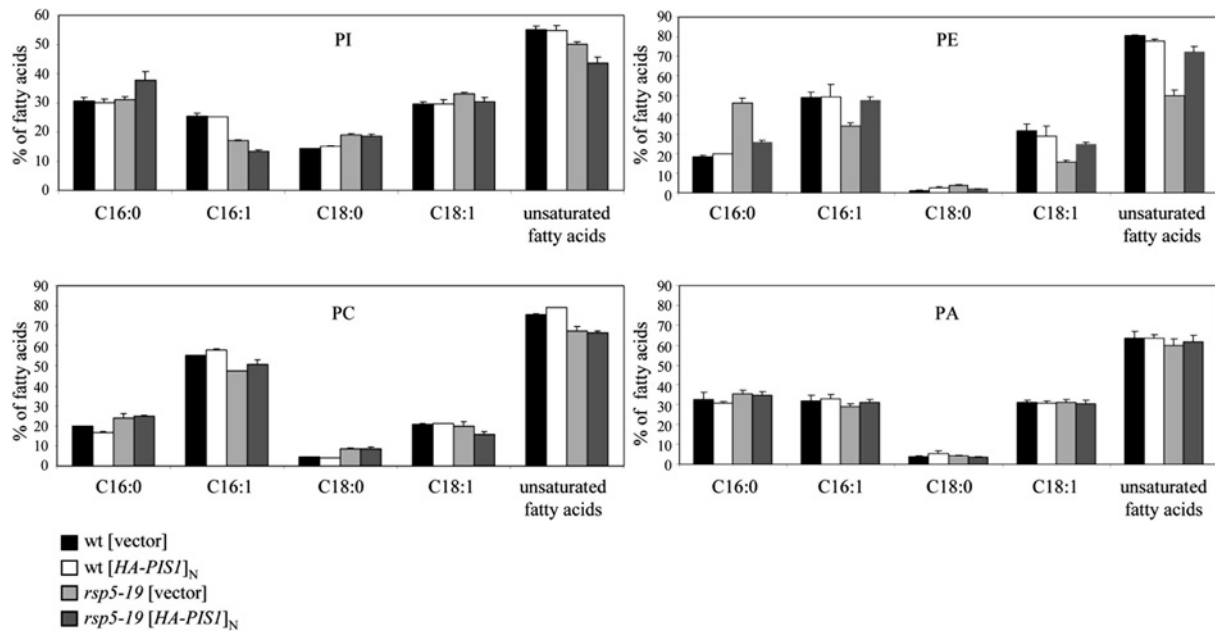


Figure 8 Overexpression of *PIS1* in the *rsp5-19* mutant promotes incorporation of saturated fatty acids into PI and restores wild-type fatty acid composition in PE

Wild-type (wt) and *rsp5-19* strains both transformed with vector or plasmid YEHA-*PIS1* were grown at 33 °C for 21 h. The fatty acid distribution in PI, PE, PC and PA were determined and are shown as a percentage of total fatty acids in the indicated phospholipid. Values are means \pm S.D. for at least 3 independent experiments. Statistical significance of differences was examined by ANOVA ($P < 0.05$, $n = 3$).

with wild-type). This increase is accompanied by the induction of *INO1* (2.5-fold), encoding Ino1p. Ino1p is involved in the synthesis of inositol-1-phosphate which after dephosphorylation serves as the Pis1p substrate in PI synthesis. Altogether, these results suggest that high levels of Pis1p (*PIS1* expressed from a multi- but not a single-copy plasmid) and PI synthesis are essential for the suppression of *rsp5* mutations.

Direct involvement of Rsp5p in *INO1* and *PIS1* regulation remains an open question. *INO1* is a highly regulated gene, the regulation of which has a major impact on phospholipid metabolism [2]. It is regulated by the Ino2p–Ino4p transcription factor complex and the Opi1 repressor in response to inositol and choline. The metabolic signal for this regulatory event is the level of PA, a common precursor molecule for the biosynthesis of phospholipids, which is detected by Opi1p in the endoplasmic reticulum [42,43]. A drop in the PA level causes Opi1p to translocate to the nucleus and perform its repressor function. In our experiments, we observed an upregulation of *INO1* under conditions in which inositol was absent from the medium. This could cause an increase in inositol levels in *rsp5* cells. Similarly, other genes that are dependent on the Ino2p–Ino4p complex and affect choline levels, might also be upregulated, but this requires further studies. Although most phospholipid biosynthetic genes are regulated at the transcriptional level in response to inositol and choline, *PIS1* is insensitive to their presence in the medium [26,44]. *PIS1* is a modestly regulated gene; its transcription is increased (2–2.5)-fold at most in various mutants and growth conditions [27,45]. A recent genomic screen aimed at characterizing mutations that affect *PIS1* expression identified 120 genes [45]. Among them were genes involved in chromatin silencing, DNA repair and many *pex* mutant genes which alter peroxisome biogenesis and function [45]. This indicated that changes within peroxisomes, potentially due to alteration in fatty acid metabolism,

result in the fluctuation of *PIS1* expression. *RSP5* was not recovered from this screen, which was restricted to non-essential genes. Thus although Rsp5 ubiquitin ligase is known to regulate the transcriptional regulators Spt23p, Mga2p and Gln3p [11,17,46], nothing is known about its effects on transcription factors that regulate *PIS1* or *INO1* expression. Therefore it is possible that the induction of *INO1* and *PIS1* gene expression in *rsp5* mutants is caused by the prevention of unsaturated fatty acids synthesis rather than by a direct regulatory effect of Rsp5p.

A high flux in phospholipid synthesis towards PI formation is probably essential for the suppression of the *rsp5* mutant, yet an increased PI level does not alone account for suppression. Indeed, despite a significant increase in Pis1p level (1.5-fold in the *rsp5-19* strain, 6-fold in the wild-type strain overexpressing *PIS1* and 9-fold in the *rsp5-19* strain overexpressing *PIS1*), the overall level of PI did not change proportionally in *rsp5* strains. However, it is noteworthy that in all these strains, the amount of PI was increased at the expense of PE.

A possible explanation for *rsp5-19* suppression by *PIS1* overexpression came from analysis of the fatty acyl content of the main phospholipids. The percentage of unsaturated/saturated fatty acids incorporated into PE was 80 % in wild-type and 50 % in the *rsp5-19* mutant at restrictive temperatures. Upon overexpression of *PIS1* this ratio in *rsp5-19* was restored to that observed in the wild-type strain. Concomitantly, saturated fatty acids preferentially substituted PI, facilitating safe storage of the detrimental excess of saturated fatty acids. In addition to inositol, Pis1p requires CDP-DAG as a substrate and competes for it with PS synthase (Cho1p) which supplies PS for synthesis of PE and PC [2]. It is known that PI is the most saturated phospholipid [34,47] and it was postulated that Pis1p has a higher affinity for CDP-DAG when esterified with saturated fatty acids, than with unsaturated fatty acids [47]. Therefore a significant increase in the

level of *Pis1p* may result in specific rerouting of CDP-DAG with saturated fatty acids towards PI. Under these conditions, relatively more CDP-DAG esterified with unsaturated fatty acids could be preferentially incorporated into PS and further into PE, restoring the unsaturation/saturation ratio of this latter phospholipid. Indeed, it has already been postulated that PI may act as a buffer for saturated fatty acids in conditions of impaired unsaturated fatty acid synthesis i.e. haem depletion [34]. These results suggest that *PIS1* overexpression, may improve the growth of *rsp5* mutants possibly by preventing a deleterious accumulation of saturated fatty acids in PE which in the wild-type strain is esterified to 80 % with unsaturated fatty acyl chains [34,47]. Moreover, decreasing the unsaturation/saturation ratio may well be detrimental to several essential cellular processes in which PE is known to be involved, such as mitochondrial integrity and function [48], membrane protein topology [49], or association of proteins into lipid rafts [50].

Finally, it is noteworthy that PI is an intermediate in the biosynthetic pathways of essential molecules, such as sphingolipids and PI phosphates, which play important regulatory roles in the cell and that could be changed in *rsp5* mutants and upon *PIS1* overexpression. Consistently, the C2 domain of the Rsp5p ubiquitin ligase binds phosphoinositides. Mutations in this domain that inhibit phosphoinositide-binding abolish interactions of Rsp5p with specific endosomal membranes with, as a result, perturbation of multivesicular body sorting of the biosynthetic cargo [16]. However, *PIS1* suppression of *rsp5* mutations by an enhanced level of phosphoinositides is not the most likely hypothesis. Indeed, the *rsp5* mutant, devoid of the C2 domain is fully viable, suggesting that binding of phosphoinositide is not required for the essential functions of Rsp5p [16]. These various aspects, which should provide additional information about the link between Rsp5p and lipid homeostasis, are presently under investigation in our respective laboratories.

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